Example 1: Cloning and expression of the hepatitis C virus E1 protein

1. Construction of vaccinia virus recombination vectors

The pgptATA18 vaccinia recombination plasmid is a modified version of pATA18 (Stunnenberg et al, 1988) with an additional insertion containing the E. coli xanthine guanine phosphoribosyl transferase gene under the control of the vaccinia virus I3 intermediate promoter (Figure 1). The plasmid pgsATA18 was constructed by inserting an oligonucleotide linker with SEQ ID NO 1/94, containing stop codons in the three reading frames, into the Pst I and HindIII-cut pATA18 vector. This created an extra Pac I restriction site (Figure 2). The original HindIII site was not restored.

Oligonucleotide linker with SEQ ID NO 1/94:

```
5'      G GCATGC AAGCTT AATTAATT      3'
3' ACGTC CGTACG TTCGAA TTAATTAA TCGA  5'
   PstI  SphI  HindIII  Pac I (HindIII)
```

In order to facilitate rapid and efficient purification by means of Ni^{2+} chelation of engineered histidine stretches fused to the recombinant proteins, the vaccinia recombination vector pMS66 was designed to express secreted proteins with an additional carboxy-terminal histidine tag. An oligonucleotide linker with SEQ ID NO 2/95, containing unique sites for 3 restriction enzymes generating blunt ends (Sma I, Stu I and Pml I/Bbr PI) was synthesized in such a way that the carboxy-terminal end of any cDNA could be inserted in frame with a sequence encoding the protease factor Xa cleavage site followed by a nucleotide sequence encoding 6 histidines and 2 stop codons (a new Pac I restriction site was also created downstream the 3'end). This oligonucleotide with SEQ ID NO 2/95 was introduced between the Xma I and Pst I sites of pgptATA18 (Figure 3).

Oligonucleotide linker with SEQ ID NO 2/95:

```
'5' CCGGG GAGGCCTGCACGTGATCGAGGGCAGACACCATCACCACCATCACTAATAGTTAATTAA CTGCAG
3'     C CTCCGGACGTGCACTAGCTCCCGTCTGTGGTAGTGGTGGTAGTGATTATCAATTAATT G
   XmaI                                                          PstI
```

FIGS. 35B–1 to 35B–8: Antibody levels to the different HCV antigens (NS4, NS5, E1 and E2) for NR and LTR followed during treatment and over a period of 6 to 12 months after treatment determined by means of the LIAscan method. The avergae vallues are indicated by the curve with the open squares.

FIGS. 36A and 36B: Average E1 antibody (E1Ab) and E2 antibody (E2Ab) levels in the LTR and NR groups.

FIGS. 37A–D: Averages E1 antibody (E1Ab) levels for non-responders (NR) and long term responders (LTR) for type 1b and type 3a.

FIG. 38: Relative map positions of the anti-E2 monoclonal antibodies.

FIG. 39: Partial deglycosylation of HCV E1 envelope protein. The lysate of vvHCV10A-infected RK13 cells were incubated with different concentrations of glycosidases according to the manufacturer's instructions. Right panel: Glycopeptidase F (PNGase F). Left panel: Endoglycosidase H (Endo H).

FIG. 40: Partial deglycosylation of HCV E2 envelope proteins. The lysate of vvHCV64-infected (E2) and vvHCV41-infected (E2s)RK13 cells were incubated with different concentrations of Glycopeptidase F (PNGase F) according to the manufacturer's instructions.

FIG. 41: In vitro mutagenesis of HCV E1 glycoproteins. Map of the mutated sequences and the creation of new restriction sites.

FIG. 42A: In vitro mutagenesis of HCV E1 glycoprotein (part 1). First step of PCR amplification.

FIG. 42B: In vitro mutagensis of HCV E1 glycoprotein (part 2). Overlap extension and nested PCR.

FIG. 43: In vitro mutagesesis of HCV E1 glycoproteins. Map of the PCR mutated fragments (GLY-# and OVR-#) synthesized during the first step of amplification.

FIG. 44A: Analysis of E1 glycoprotein mutants by Western blot expressed in HeLa (left) and RK13 (right) cells. Lane 1: wild type VV (vaccinia virus), Lane 2: original E1 protein (vvHCV-10A), Lane 3: E1 mutant Gly-1 (vvHCV-81), Lane 4: E1 mutant Gly-2 (vvHCV-82), Lane 5: E1 mutant Gly-3 (vvHCV-83), Lane 6: E1 mutant Gly-4 (vvHCV-84), Lane 7: E1 mutant Gly-5 (vvHCV-85), Lane 8: E1 mutant Gly-6 (vvHCV-86).

FIG. 44B: Analysis of E1 glycosylation mutant vaccinia viruses by PCR amplification/restriction. Lane 1: E1 (vvHCV-10A), BsoE I, Lane 2: E1.GLY- 1 (vvHCV-81), BspE I, Lane 4: E1 (vvHCV- 10A), Sac I, Lane 5: E1.GLY-2 (vvHCV-82), Sac I, Lane 7: E1 (vvHCV-10A), Sac I, Lane 8: E1 .GLY-3 (vvHCV-83), Sac I, Lane 10: E1 (vvHCV-10A), Stu I, Lane 11: E1.GLY-4 (vvHCV-84), Stu I, Lane 13: E1 (vvHCV-10A), Sma I, Lane 14: E1.GLY-5 (vvHCV-85), Sma I, Lane 16: E1 (vvHCV-10A), Stu I, Lane 17: E1.GLY-6 (vvHCV-86), Stu I, Lane 3-6-9-12-15: Low Molecular Weight Marker, pBluescript SK+, Msp I.

FIG. 45: SDS polyacrylamide gel electrophoresis of recombinant E2 expressed in *S. cerevisiae*. Innoculates were grown in leucine selective medium for 72 hrs. and diluted 1/15 in complete medium. After 10 days of culture at 28° C., medium samples were taken. The equivalent of 200 µl of culture supernatant concentrated by speedvac was loaded on the gel. Two independent transformants were analysed.

FIG. 46: SDS polyacrylamide gel electrophoresis of recombinant E2 expressed in a glycosylation deficient *S. cerevisiae* mutant. Innoculae were grown in leucine selective medium for 72 hrs. and diluted 1/15 in complete medium. After 10 days of culture at 28° C., medium samples were taken. The equivalent of 350 µl of culture supernatant, concentrated by ion exchange chromatography, was loaded on the gel.

Table 1 : Features of the respective clones and primers used for amplification for constructing the different forms of the E1 protein as despected in Example 1.

Table 2 : Summary of Anti-E1 tests

Table 3 : Synthetic peptides for competition studies

Table 4: Changes of envelope antibody levels over time.

Table 5: Difference between LTR and NR

Table 6: Competition experiments between murine E2 monoclonal antibodies

Table 7: Primers for construction of E1 glycosylation mutants

Table 8: Analysis of E1 glycosylation mutants by ELISA

EXAMPLE 1

Cloning and Expression of the Hepatitis C Virus E1 Protein

1. Construction of Vaccinia Virus Recombination vectors

The pgptATA18 vaccinia recombination plasmid is a modified version of pATA18 (Stunnenberg et al, 1988) with an additional insertion containing the *E. coli* xanthine guanine phosphoribosyl transferase gene under the control of the vaccinia virus 13 intermediate promoter (FIG. 1). The plasmid pgsATA18 was constructed by inserting an oligonucleotide linker with SEQ ID NO 1/94, containing stop codons in the three reading frames, into the Pst I and HindIII-cut pATA18 vector. This created an extra Pac I restriction site (FIG. 2). The original HindIII site was not restored.

Oligonucleotide linker with SEQ ID NO 1/94:

```
5'      G GCATGC AAGCTT AATTAATT          3'

3' ACGTC CGTACG TTCGAA TTAATTAA TCGA    5'
   PstI  SphI  HindIII  Pac I (HindIII)
```

In order to facilitate rapid and efficient purification by means of Ni^{2}- chelation of engineered histidine stretches fused to the recombinant proteins, the vaccinia recombination vector pMS66 was designed to express secreted proteins with an additional carboxy-terminal histidine tag. An oligonucleotide linker with SEQ ID NO 2/95, containing unique sites for 3 restriction enzymes generating blunt ends (Sma i, Stu I and PmlI/BbrPI) was synthesized in such a way that the carboxy-terminal end of any cDNA could be inserted in frame with a sequence encoding the protease factor Xa cleavage site followed by a nucleotide sequence encoding 6 histidines and 2 stop codons (a new Pac I restriction site was also created downstream the 3' end). This oligonucleotide

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with SEQ ID NO 2/95 was introduced between the Xma I and Pst I sites of pgptATA18 (FIG. 3).

Oligonucleotide linker with SEQ ID NO 2/95:

```
Oligonucleotide linker with SEQ ID NO 2/95:
'5'   CCGGG GAGGCCTGCACGTGATCGAGGGCAGACACCATCACCACCATCACTAATAGTTAATTAA CTGCAJ

3'      C CTCCGGACGTGCACTAGCTCCCGTCTGTGGTAGTGGTGGTAGTGATTATCAATTAATT G
    XmaI                                                              PstI
```

EXAMPLE 2

Construction of HCV Recombinant Plasmids

2.1. Constructs Encoding Different Forms of the E1 Protein

Polymerase Chain Reaction (PCR) products were derived from the serum samples by RNA preparation and subsequent reverse-transcription and PCR as described previously (Stuyver et al., 1993b). Table 1 shows the features of the respective clones and the primers used for amplification. The PCR fragments were cloned into the Sma I-cut pSP72 (Promega) plasmids. The following clones were selected for insertion into vaccinia reombination vectors: HCCl9A (SEQ ID NO 3), HCCl1 OA (SEQ ID NO 5), HCCl11A (SEQ ID NO 7), HCCl12A (SEQ ID NO 9), HCCl13A (SEQ ID NO 11), and HCCl17A (SEQ ID NO 13) as depicted in FIG. 21. cDNA fragments containing the E1-coding regions were cleaved by EcoRI and HindIII restriction from the respective pSP72 plasmids and inserted into the EcoRl/Hindll-cut pgptATA-18 vaccinia recombination vector (described in example 1), downstream of the 11K vaccinia virus late promoter. The respective plasmids were designated pvHCV-9A, pvHCV-10A, pvHCV-11A, pvHCV-12A, pvHCV-13A and pvHCV-17A, of which pvHCV-11A is shown in FIG. 4.

2.2. Hydrophobic Region E1 Deletion Mutants

Clone HCCl37, containing a deletion of codons Asp264 to Val287 (nucleotides 790 to 861, region encoding hydrophobic domain 1) was generated as follows: 2 PCR fragments were generated from clone HCCl10A with primer sets HCPr52 (SEQ ID NO 16)/HCPr107 (SEQ ID NO 19) and HCPr108 (SEQ ID NO 20)/HCPR54 (SEQ ID NO 18). These primers are shown in FIG. 21. The two PCR fragments were purified from agarose gel after electrophoresis and 1 ng of each fragment was used together as template for PCR by means of primers HCPr52 (SEQ ID NO 16) and HCPr54 (SEQ ID NO 18). The resulting fragment was cloned into the Sma I-cut pSP72 vector and clones containing the deletion were readily identified because of the deletion of 24 codons (72 base pairs). Plasmid pSP72HCC137 containing clone HCC137 (SEQ ID 15) was selected. A recombinant vaccinia plasmid containing the full-length E1 cDNA lacking hydrophobic domain I was constructed by inserting the HCV sequence surrounding the deletion (fragment cleaved by Xma I and BamH I from the vector pSP72-HCCl37) into the Xma I-BamHI sites of the vaccinia plasmid pvHCV-10A. The resulting plasmid was named pvHCV-37. After confirmatory sequencing, the amino-terminal region containing the internal deletion was isolated from this vector pvHCV-37 (cleavage by EcoR I and BstE II) and reinserted into the Eco RI and Bst Ell-cut pvHCV-11A plasmid. This construct was expected to express an E1 protein with both hydrophobic domains deleted and was named pvHCV-38. The E1-coding region of clone HCCl38 is represented by SEQ ID NO 23.

As the hydrophilic region at the E1 carboxyterminus (theoretically extending to around amino acids 337–340) was not completely included in construct pvHCV-38, a larger E1 region lacking hydrophobic domain I was isolated from the pvHCV-37 plasmid by EcoRl/Bam HI cleavage and cloned into an EcoRII/BamHI-cut pgsATA-18 vector. The resulting plasmid was named pvHCV-39 and contained clone HCCl39 (SEQ ID NO 25). The same fragment was cleaved from the pvHCV-37 vector by BamH I (of which the sticky ends were filled with Klenow DNA Polymerase I (Boehringer)) and subsequently by EcoR1 (5' cohesive end). This sequence was inserted into the EcoRI and Bbr PI-cut vector pMS-66. This resulted in clone HCCl40 (SEQ ID NO 27) in plasmid pvHCV-40, containing a 6 histidine tail at its carboxy-terminal end.

2.3. E1 of Other Genotypes

Clone HCCl62 (SEQ ID NO 29) was derived from a type 3a-infected patient with chronic hepatitis C (serum BR36, clone BR36-9-13, SEQ ID NO 19 in WO 94/25601, and see also Stuyver et al. 1993a) and HCCl63 (SEQ ID NO 31) was derived from a type 5a-infected child with post-transfusion hepatitis (serum BE95, clone PC-4-1, SEQ ID NO 45 in WO 94/25601).

2.4. E2 Constructs

The HCV E2 PCR fragment 22 was obtained from serum BE11 (genotype 1b) by means of primers HCPr109 (SEQ ID NO 33) and HCPr72 (SEQ ID NO 34) using techniques of RNA preparation, reverse-transcription and PCR, as described in Stuyver et al., 1993b, and the fragment was cloned into the Sma I-cut pSP72 vector. Clone HCCl22A (SEQ ID NO 35) was cut with NcoI/AlwNI or by BamHI/AlwNI and the sticky ends of the fragments were blunted (NcoI and BamHI sites with Klenow DNA Polymerase I (Boehringer), and AlwNI with T4 DNA polymerase (Boehringer)). The BamHI/AlwNI cDNA fragment was then inserted into the vaccinia pgsATA-18 vector that had been linearized by EccRI and Hind III cleavage and of which the cohesive ends had been filled with Klenow DNA Polymerase (Boehringer). The resulting plasmid was named pvHCV-41 and encoded the E2 region from amino acids Met347 to Gln673, including 37 amino acids (from Met347 to Gly383) of the E1 protein that can serve as signal sequence. The same HCV cDNA was inserted into the EcoR I and Bbr PI-cut vector pMS66, that had subsequently been blunt ended with Klenow DNA Polymerase. The resulting plasmid was named pvHCV-42 and also encoded amin acids 347 to 683. The NcoI/AlwNI fragment was inserted in a similar way into the same sites of pgsATA-18 (pvHCV-43) or pMS-66 vaccinia vectors (pvHCV-44). pvHCV-43 and pvHCV-44 encoded amino acids 364 to 673 of the HCV polyprotein, of which amino acids 364 to 383 were derived from the natural carboxyterminal region of the E1 protein encoding the signal sequence for E2, and amino acids 384 to 673 of the mature E2 protein.

2.5. Generation of Recombinant HCV-Vaccinia Viruses

Rabbit kidney RK13 cells (ATCC CCL 37), human osteosarcoma 143B thymidine kinase deficient (TK-) (ATCC CRL 8303), HeLa (ATCC CCL 2), and Hep G2 (ATCC HB 8065) cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, Md., USA).

ible 4. Change of Envelope Antibody levels over time (complete study, 28 patients)

ilcoxon Signed nk test (P values)	E1Ab NR All	E1Ab NR type 1b	E1Ab NR type 3a	E1Ab LTR All	E1Ab LTR type 1b	E1Ab LTR type 3a	E2Ab NR All	E1Ab LTR All
id of therapy*	0.1107	0.2604	0.285	0.0058**	0.043**	0.0499**	0.0186**	0.0640
months follow up*	0.86	0.7213	0.5930	0.0047**	0.043**	0.063	0.04326	0.0464**
2 months follow up*	0.7989	0.3105	1	0.0051**	0.0679	0.0277**	0.0869	0.0058**

Data were compared with values obtained at initiation of therapy
P values < 0.05

TABLE 1-continued

Recombinant vaccinia plasmids and viruses

Plasmid name	Name	cDNA subclone construction	Length (nt/aa)	Vector used for insertion
pvHCV-65	E1-E2	BamH I - Hind III	2072/691	pvHCV-10A
pvHCV-66	CORE-E1-E2	BamH I - Hind III	2427/809	pvHCV-33
pvHCV-81	E1*-GLY 1	EcoRI - BamH I	783/262	pvHCV-10A
pvHCV-82	E1*-GLY 2	EcoRI - BamH I	783/262	pvHCV-10A
pvHCV-83	E1*-GLY 3	EcoRI - BamH I	783/262	pvHCV-10A
pvHCV-84	E1*-GLY 4	EcoRI - BamH I	783/262	pvHCV-10A
pvHCV-85	E1*-GLY 5	EcoRI - BamH I	783/262	pvHCV-10A
pvHCV-86	E1*-GLY 6	EcoRI - BamH I	783/262	pvHCV-10A

nt: nucleotide
aa: aminoacid
Kl: Klenow DNA Pol filling
T4:T4 DNA Pol filling
Position: aminoacid positionin the HCV polyprotein sequence

TABLE 2

Summary of anti-E1 tests
S/N ± SD (mean anti-E1 titer)

	Start of treatment	End of treatment	Follow-up
LTR	6.94 ± 2.29 (1:3946)	4.48 ± 2.69 (1:568)	2.99 ± 2.69 (1:175)
NR	5.77 ± 3.77 (1:1607)	5.29 ± 3.99 (1:1060)	6.08 ± 3.73 (1:1978)

LTR: Long-term, sustained response for more than 1 year
NR: No response, response with relapse, or partial response

TABLE 3

Synthetic peptides for competition studies

PROTEIN	PEPTIDE	AMINO ACID SEQUENCE	POSITION	SEQ ID NO
E1	E1-31	LLSCLTVPASAYQVRNSTGL	181–200	56
	E1-33	QVRNSTGLYHVTNDCPNSSI	193–212	57
	E1-35	NDCPNSSIVYEAHDAILHTP	205–224	58
	E1-35A	SNSSIVYEAADMIMHTPGCV	208–227	59
	E1-37	HDAILHTPGCVPCVREGNVS	217–236	60
	E1-39	CVREGNVSRCWVAMTPTVAT	229–248	61
	E1-41	AMTPTVATRDGKLPATQLRR	241–260	62
	E1-43	LPATQLRRHIDLLVGSATLC	253–272	63
	E1-45	LVGSATLCSALYVGDLCGSV	265–284	64
	E1-49	QLFTFSPRRHWTTQGCNCSI	289–308	65
	E1-51	TQGCNCSIYPGHITGHRMAW	301–320	66
	E1-53	ITGHRMAWDMMMNWSPTAAL	313–332	67
	E1-55	NWSPTAALVMAQLLRIPQAI	325–344	68
	E1-57	LLRJPQAILDMIAGAHWGVL	337–356	69
	E1-59	AGAHWGVLAGIAYFSMVGNM	349–368	70

TABLE 3-continued

Synthetic peptides for competition studies

PROTEIN	PEPTIDE	AMINO ACID SEQUENCE	POSITION	SEQ ID NO
	E1-63	VVLLLFAGVDAETIVSGGQA	373–392	71
E2	E2-67	SGLVSLFTPGAKQNJQLINT	397–416	72
	E2-69	QNIQLINTNGSWHINSTALN	409–428	73
	E2-$3B	LNCNESLNTGWWLAGLIYQHK	427–446	74
	E2-$1B	AGLIYQHKFNSSGCPERLAS	439–458	75
	E2-1B	GCPERLASCRPLTDFDQGWG	451–470	76
	E2-3B	TDFDQGWGPISYANGSGPDQ	463–482	77
	E2-5B	ANGSGPDQRPYCWHYPPKPC	475–494	78
	E2-7B	WHYPPKPCGIVPAKSVCGPV	487–506	79
	E2-9B	AKSVCGPVYCFTPSPVVVGT	499–518	80
	E2-11B	PSPVVVGTTDRSGAPTYSWG	511–530	81
	E2-13B	GAPTYSWGENDTDVFVLNNT	523–542	82
	E2-17B	GNWFGCTWMNSTGFTKVCGA	547–566	83
	E2-19B	GFTKVCGAPPVCIGGAGNNT	559–578	84
	E2-21	IGGAGNNTLHCPTDCFRKHP	571–590	85
	E2-23	TDCFRKHPDATYSRCGSGPW	583–602	86
	E2-25	SRCGSGPWITPRCLVDYPYR	595–614	87
	E2-27	CLVDYPYRLWHYPCTNYTI	607–626	88
	E2-29	PCTINYTIFKIRMYVGGVEH	619–638	89
	E2-31	MYVGGVEHRLEAACNWTPGE	631–650	90
	E2-33	ACNWTPGERCDLEDRDRSEL	643–662	91
	E2-35	EDRDRSELSPLLLTTTQWQV	655–674	92

TABLE 4

Change of Envelope Antibody levels over time (complete study, 28 patients)

Wilcoxon Signed Rank test (P values)	E1Ab NR All	E1Ab NR type 1b	E1Ab NR type 3a	E1Ab LTR II	E1Ab LTR type 1b	E1Ab LTR type 3a	E2Ab NR All	E1Ab LTR All
End of therapy*	0.1167	0.2604	0.285	0.0058**	0.043**	0.0499**	0.0186**	0.0640
6 months follow up*	0.86	0.7213	0.5930	0.0047**	0.043**	0.063	0.04326	0.0464**
12 months follow up*	0.7989	0.3105	1	0.0051**	0.0679	0.0277**	0.0869	0.0058**

*Data were compared with values obtained at initiation of therapy
**P values < 0.05

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Table 8. Analysis of E1 glycosylation mutants by ELISA

SERUM

	1	2	3	4	5	6	7	8	9	10	11	12		
iLY1	1.802462	2.120971	1.403871	1.205597	2.120191	2.866913	1.950345	1.866103	1.730193	2.468162	1.220654	1.629403		
iLY2	2.400795	1.76818	2.325495	2.639308	2.459019	5.043993	2.146302	1.595477	1.688973	2.482212	1.467502	2.070524		
iLY3	1.642718	1.715477	2.261648	2.354748	1.591818	4.833742	1.96692	1.482099	1.602222	2.191558	1.464216	1.721164		
iLY4	2.578154	3.824038	3.874605	1.499387	3.15	4.71302	4.198751	3.959542	3.710507	5.170841	4.250784	3.955153		
iLY5	2.482051	1.793761	2.409344	2.627358	1.715311	4.964765	2.13912	1.576336	1.708937	3.021807	1.562092	2.07278		
iLY6	2.031487	1.495737	2.131613	2.527925	2.494833	4.784027	2.02069	1.496489	1.704978	2.677757	1.529608	1.744221		
1	2.828205	2.227036	2.512792	2.790881	3.131579	4.869128	2.287753	1.954198	1.805556	2.616822	1.55719	2.593086		
	13	14	15	16	17	18	19	20	21	22	23	24	Sum S/N	Average S/N
iLY1	5.885561	3.233604	3.763498	1.985105	2.317721	6.675179	1.93476	2.47171	4.378633	1.188748	2.150889	1.706992	59.88534	2.495223
iLY2	7.556882	2.567813	3.621928	3.055649	2.933792	7.65433	2.127712	2.921288	4.680101	1.150781	1.661914	1.632785	69.65243	2.902185
iLY3	7.930538	2.763055	3.016099	2.945628	2.515305	5.775357	1.980185	2.557384	4.268633	0.97767	1.336775	1.20376	62.09872	2.587447
iLY4	8.176816	6.561122	5.707668	5.684498	5.604813	6.4125	3.013321	3.002535	4.293038	2.393011	3.68213	2.481585	102.6978	4.279076
iLY5	8.883408	2.940334	3.125561	3.338912	2.654224	5.424107	2.442804	3.126761	4.64557	1.153656	1.817901	1.638211	69.26511	2.886046
iLY6	8.005561	2.499952	2.621704	2.572385	2.363301	5.194107	1.506716	2.665433	2.781063	1.280743	1.475062	1.716423	61.32181	2.555075
1	8.825112	3.183771	3.067265	3.280335	2.980354	7.191964	2.771218	3.678068	5.35443	1.167286	2.083333	1.70252	76.54068	3.189195

SERUM

	1	2	3	4	5	6	7	8	9	10	11	12		
/E1	0.637316	0.952374	0.55069	0.431977	0.677036	0.580794	0.852516	0.954961	0.950261	0.94319	0.783882	0.620171		
/E1	0.848076	0.793961	0.925463	0.94569	0.785233	1.035913	0.93017	0.816436	0.935431	0.94856	0.942455	0.798232		
/E1	0.580834	0.770296	0.900053	0.84373	0.508312	0.992733	0.859761	0.758418	0.867385	0.837488	0.940294	0.663547		
/E1	0.911587	1.717097	1.541952	0.537245	1.005882	0.967939	1.035317	2.026172	2.05505	1.976	2.72978	1.524798		
/E1	0.877607	0.805447	0.958831	0.941408	0.547746	1.019642	0.935031	0.806641	0.946488	1.154762	1.003148	0.799102		
/E1	0.718296	0.671626	0.848305	0.90578	0.796669	0.902522	0.883264	0.765781	0.944294	1.023286	0.982288	0.672435		
	13	14	15	16	17	18	19	20	21	22	23	24	Sum E1/GLY#	Average E1/GLY#
/E1	0.644248	1.015852	1.226988	0.605153	0.777666	0.928144	0.698162	0.672013	0.817759	1.018386	1.036267	0.957628	19.36524	0.806885
/E1	0.85627	0.806469	1.180833	0.931505	0.984377	1.064289	0.76779	0.794245	0.874061	0.98586	0.797719	0.915998	21.67384	0.903077
/E1	0.898633	0.867856	0.983319	0.897966	0.843962	0.803029	0.714554	0.695306	0.797215	0.837558	0.641652	0.675314	19.19921	0.799967
/E1	0.92654	2.060802	1.860833	1.732902	1.880587	0.89162	1.376045	0.816335	0.801773	2.050064	1.767422	1.392178	36.38592	1.51608
/E1	1.006606	0.923538	1.019006	1.017857	0.890574	0.75419	0.881491	0.850109	0.867612	0.988323	0.872593	0.919042	21.70679	0.907783
/E1	0.907134	0.785217	0.854737	0.784184	0.79296	0.72221	0.543702	0.724683	0.519395	1.097197	0.70803	0.962919	19.59691	0.816538

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TABLE 8

Analysis of E1 glycosylation mutants by ELISA

	SERUM													
	1	2	3	4	5	6	7	8	9	10	11	12		
SN GLY 1	1.802462	2.120971	1.403871	1.205597	2.120191	2.866913	1.950345	1.866183	1.730193	2.468162	1.220654	1.629403		
SN GLY 2	2.400795	1.76818	2.325495	2.639308	2.459019	5.043993	2.146302	1.595477	1.688973	2.482212	1.467582	2.070524		
SN GLY 3	1.642718	1.715477	2.261646	2.354748	1.591818	4.833742	1.96692	1.482099	1.602222	2.191558	1.464216	1.721164		
SN GLY 4	2.578154	3.824038	3.874605	1.499387	3.15	4.71302	4.198751	3.959542	3.710507	5.170841	4.250784	3.955153		
SN GLY 5	2.482051	1.793761	2.409344	2.627358	1.715311	4.964765	2.13912	1.576336	1.708937	3.021807	1.562092	2.07278		
SN GLY 6	2.031487	1.495737	2.131613	2.527925	2.494833	4.784027	2.02069	1.496489	1.704976	2.677757	1.529608	1.744221		
SN E1	2.828205	2.227036	2.512792	2.790881	3.131579	4.869128	2.287753	1.954198	1.805556	2.616822	1.55719	2.593886		
	13	14	15	16	17	18	19	20	21	22	23	Sum 24	Average S/N	S/N
SN GLY 1	5.685561	3.233684	3.763498	1.985105	2.317721	6.675179	1.93476	2.47171	4.378633	1.188748	2.158889	1.786992	59.88534	2.495223
SN GLY 2	7.556682	2.567613	3.621928	3.055649	2.933792	7.65433	2.127712	2.921288	4.680101	1.158781	1.661914	1.632785	69.65243	2.982185
SN GLY 3	7.930538	2.763055	3.016099	2.945628	2.515305	5.775357	1.980185	2.557384	4.268633	0.97767	1.336775	1.20376	62.89872	2.587447
SN GLY 4	8.176816	6.561122	5.707668	5.684498	5.604813	6.4125	3.813321	3.002535	4.293038	2.393011	3.68213	2.481585	102.6978	4.279076
SN GLY 5	8.883408	2.940334	3.125561	3.338912	2.654224	5.424107	2.442804	3.126761	4.64557	1.153656	1.817901	1.638211	69.26511	2.886846
SN GLY 6	8.005561	2.499952	2.621704	2.572385	2.363301	5.194107	1.586716	2.665433	2.781063	1.280743	1.475862	1.716423	61.32181	2.555075
SN E1	8.825112	3.183771	3.067265	3.280335	2.980354	7.191964	2.771218	3.678068	5.35443	1.167286	2.083333	1.78252	76.54068	3.189195

	SERUM													
	1	2	3	4	5	6	7	8	9	10	11	12		
GLY 1/E1	0.637316	0.952374	0.55869	0.431977	0.677036	0.588794	0.852516	0.954961	0.958261	0.94319	0.783882	0.628171		
GLY 2/E1	0.848876	0.793961	0.925463	0.94569	0.785233	1.035913	0.93817	0.816436	0.935431	0.94856	0.942455	8.798232		
GLY 3/E1	0.580834	0.770296	0.900053	0.84373	0.508312	0.992733	0.859761	0.758418	0.887385	0.837488	0.940294	8.663547		
GLY 4/E1	0.911587	1.717097	1.541952	0.537245	1.005882	0.967939	1.835317	2.026172	2.05505	1.976	2.72978	1.524798		
GLY 5/E1	0.877607	0.805447	0.958831	0.941488	0.547746	1.019642	0.935031	0.806641	0.946488	1.154762	1.883148	8.799182		
GLY 6/E1	0.718296	0.671626	0.848305	0.90578	0.796669	0.982522	0.883264	0.765781	0.944294	1.023286	0.982288	8.672435		
	13	14	15	16	17	18	19	20	21	22	23	24	Sum E1/GLY #	Average E1/GLY #
GLY 1/E1	0.644248	1.815652	1.226988	0.605153	0.777666	0.928144	0.698162	0.672013	0.817759	1.018386	1.836267	0.957628	19.36524	0.806885
GLY 2/E1	8.85627	6.806469	1.188833	0.931505	8.984377	1.064289	8.76779	0.794245	0.874061	0.98586	8.797719	8.915998	21.67384	0.903077
GLY 3/E1	0.898633	0.867856	8.983319	0.897966	0.843962	0.883029	0.714554	0.695306	0.797215	0.837558	0.641652	0.675314	19.19921	0.799967
GLY 4/E1	0.92654	2.060802	1.860833	1.732902	1.880587	0.89162	1.376045	8.816335	8.801773	2.058864	1.767422	1.392178	36.38592	1.51608
GLY 5/E1	1.006606	0.923538	1.019006	1.817857	0.890574	8.75419	0.881491	0.850109	8.867612	8.988323	0.872593	0.919042	21.78679	8.907783
GLY 6/E1	0.907134	0.785217	8.854737	0.784184	0.79296	8.72221	0.543702	8.724683	0.519395	1.097197	0.70803	0.962919	19.59691	0.816538

Table 5. Difference between LTR and NR (complete study)

Mann-Withney U test (P values)	E1Ab S/N All	E1Ab titers All	E1Ab S/N type 1b	E1Ab S/N type 3a	E2Ab S/N All
Initiation of therapy	0.0257*		0.05*	0.68	0.1078
End of therapy	0.1742				0.1295
6 months follow up	1		0.6099	0.425	0.3081
12 months follow up	0.67		0.23	0.4386	0.6629

*P values < 0.05

able 6. Competition experiments between murine E2 monoclonal antibodies

	Decrease (%) of anti-E2 reactivity of biotinylated anti-E2 mabs									
ompetitor	17H10F4D10	2F10H10	16A6E7	10D3C4	4H6B2	17C2F2	9G3E6	12D11F1	15C8C1	8G10D1H9
7H10F4D10	-	62	10	ND	11	ND	5	6	30	ND
F10H10	90	-	1	ND	30	ND	0	4	12	ND
6A6E7	ND	ND	-	ND	ND	ND	ND	ND	ND	ND
0D3C4	11	50	92	-	94	26	28	43	53	30
H6B2	ND	ND	82	ND	-	ND	ND	ND	ND	ND
7C2F2	2	ND	75	ND	56	-	11	10	0	0
G3E6	ND	ND	68	ND	11	ND	-	60	76	ND
2D11F1	ND	ND	26	ND	13	ND	ND	-	88	ND
5C8C1	ND	ND	18	ND	10	ND	ND	ND	-	ND
G10D1H9	2	2	11	ND	15	ND	67	082	81	-
ompetitor controls										
5B7A2	0	0	9	15	10	9	0	0	0	5
H6A7	0	2	0	12	8	0	0	4	0	0
3C12H9	ND	ND	2	12	ND	4	ND	ND	ND	2

ID, not done

Table 7. Primers

SEQ ID NO. 96	GPT	5'-GTTTAACCACTGCATGATG-3'
SEQ ID NO. 97	TK_n	5'-GTCCCATCGAGTGCGGCTAC-3'
SEQ ID NO. 98	GLY1	5'-CGTGACATGGTACATTCCGGACACTTGGCGCACTTCATAAGCGGA-3'
SEQ ID NO. 99	GLY2	5'-TGCCTCATACACAATGGAGCTCTGGGACGAGTCGTTCGTGAC-3'
SEQ ID NO. 100	GLY3	5'-TACCCAGCAGCGGGAGCTCTGTTGCTCCCGAACGCAGGGCAC-3'
SEQ ID NO. 101	GLY4	5'-TGTCGTGGTGGGGACGGAGGCCTGCCTAGCTGCGAGCGTGGG-3'
SEQ ID NO. 102	GLY5	5'-CGTTATGTGGCCCGGGTAGATTGAGCACTGGCAGTCCTGCACCGTCTC-3'
SEQ ID NO. 103	GLY6	5'-CAGGGCCGTTGTAGGCCTCCACTGCATCATCATATCCCAAGC-3'
SEQ ID NO. 104	OVR1	5'-CCGGAATGTACCATGTCACGAACGAC-3'
SEQ ID NO. 105	OVR2	5'-GCTCCATTGTGTATGAGGCAGCGG-3'
SEQ ID NO. 106	OVR3	5'-GAGCTCCCGCTGCTGGGTAGCGC-3'
SEQ ID NO. 107	OVR4	5'-CCTCCGTCCCCACCACGACAATACG-3'
SEQ ID NO. 108	OVR5	5'-CTA*CCCGGG*CCACATAACGGGTCACCG-3'
SEQ ID NO. 109	OVR6	5'-GGAGGCCTACAACGGCCCTGGTGG-3'
SEQ ID NO. 110	GPT-2	5'-TTCTATCGATTAAATAGAATTC -3'
SEQ ID NO. 111	TK_n-2	5'-GCCATACGCTCACAGCCGATCCC-3'

nucleotides underlined represent additional restriction site

nucleotides in bold represent mutations with respect to the original HCCI10A sequence

TABLE 5

Difference between LTR and NR (complete study)

Mann-Withney U test (P values)	E1Ab S/N All	E1Ab titers all	E1Ab S/N type 1b	E1Ab S/N type 3a	E2Ab S/N All
Initiation of therapy	0.0257*		0.05*	0.68	0.1078
End of therapy	0.1742				0.1295
6 months follow up,	1		0.6099	0.425	0.3081
12 months follow up	0.67		0.23	0.4386	0.6629

*P values < 0.05

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TABLE 6

Competition experiments between murine E2 monoclonal antibodies

	Decrease (%) of anti-E2 reactivity of biotinylated anti-E2 mabs									
	17H10F4D10	2F10H10	16A6E7	10D3C4	4H6B2	17C2F2	9G3E6	12D11F1	15C8C1	8G10D1H9
competitor										
17H10F4D10		62	10	ND	11	ND	5	6	30	ND
2F10H10	90	—	1	ND	30	ND	0	4	12	ND
16A6E7	ND	ND	—	ND	ND	ND	ND	ND	ND	ND
10D3C4	11	50	92	—	94	26	28	43	53	30
4H6B2	ND	ND	82	ND	—	ND	ND	ND	ND	ND
17C2F2	2	ND	75	ND	56	—	11	10	0	0
9G3E6	ND	ND	68	ND	11	ND	—	60	76	ND
12D11F1	ND	ND	26	ND	13	ND	ND	—	88	ND
15C8C1	ND	ND	18	ND	10	ND	ND	ND	—	ND
8G10D1H9	2	2	11	ND	15	ND	67	0.82	81	—
competitor controls										
15B7A2	0	0	9	15	10	9	0	0	0	5
5H6A7	0	2	0	12	8	0	0	4	0	0
23C12H9	ND	ND	2	12	ND	4	ND	ND	ND	2

ND, not done

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TABLE 7

Primers

SEQ ID NO. 96	GPT	5'-GTTTAACCACTGCATGATG-3'
SEQ ID NO. 97	TK_R	5'-GTCCCATCGAGTGCGGCTAC-3'
SEQ ID NO. 98	GLY1	5'-CGTGACATGGTACATTCCGGACACTTGGCGCACTTCATAAGCGGA-3'
SEQ ID NO. 99	GLY2	5'-TGCCTCATACACAATGGAGCTCTGGGACGAGTCGTTCGTGAC-3'
SEQ ID NO. 100	GLY3	5'-TACCCAGCAGCGGGAGCTCTGTTGCTCCCGAACGCAGGGCAC-3'
SEQ ID NO. 101	GLY4	5'-TGTCGTGGTGGGGACGGAGGCCTGCCTAGCTGCGAGCGTGGG-3'
SEQ ID NO. 102	GLY5	5'-CGTTATGTGGCCCGGGTAGATTGAGCACTGGCAGTCCTGCACCGTCTC-3'
SEQ ID NO. 103	GLY6	5'-CAGGGCCGTTGTAGGCCTCCACTGCATCATCATATCCCAAGC-3'
SEQ ID NO. 104	OVR1	5'-CCGGAATGTACCATGTCACGAACGAC-3'
SEQ ID NO. 105	OVR2	5'-GCTCCATTGTGTATGAGGCAGCGG-3'
SEQ ID NO. 106	OVR3	5'-GAGCTCCCGCTGCTGGGTAGCGC-3'
SEQ ID NO. 107	OVR4	5'-CCTCCGTCCCCACCACGACAATACG-3'
SEQ ID NO. 108	OVR5	5'-CTACCCGGGCCACATAACGGGTCACCG-3'
SEQ ID NO. 109	OVR6	5'-GGAGGCCTACAACGGCCCTGGTGG-3'
SEQ ID NO. 110	GPT-2	5'-TTCTATCGATTAAATAGAATTC-3'
SEQ ID NO. 111	TK_R-2	5'-GCCATACGCTCACAGCCGATCCC-3'

nucleotides underlined represent additional restriction site
nucleotides in bold represent mutations with respect to the original HCC110A sequence

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